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Peptidomic study of casein proteolysis in bovine milk by *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 / Solieri, Lisa; DE VERO, Luciana; Tagliazucchi, Davide. - In: INTERNATIONAL DAIRY JOURNAL. - ISSN 0958-6946. - 85:(2018), pp. 237-246. [10.1016/j.idairyj.2018.06.010]

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02/05/2024 06:08

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Peptidomic study of casein proteolysis in bovine milk by *Lactobacillus casei*

PRA205 and *Lactobacillus rhamnosus* PRA331

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1 **Abstract**

2 Lactobacilli contain different cell envelope proteinases (CEPs) responsible for the
3 hydrolysis of caseins and the release of various bioactive peptides. In this work, we explored the
4 CEP activity of *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 whole cells
5 towards β -, α S1-, κ - and α S2-caseins in bovine milk. Mass spectrometry analysis of fermented milk
6 hydrolysates identified a total of 331 peptides, which were mainly derived from β -caseins (59.0 and
7 60.1% for PRA205 and PRA331, respectively). The analysis of α S1-casein (f1-23) cleavage site
8 specificity congruently supports that *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 exhibited a
9 mixed-type CEP_{I/III} activity. PRA205 and PRA331 CEPs also showed cleavage site specificity
10 toward β -casein, preferentially. These CEPs cleaved the peptide bond preferentially when
11 hydrophobic or negatively charged amino acids were present. 13.5% and 13.7% of peptides released
12 by *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 CEPs were found to have 100% homology with
13 previously identified bioactive peptides.

14 **1. Introduction**

15 Food intake with the goal of improving human health is an ongoing focus for research.
16 Recommendations for the consumption of certain nutritious fermented foods date back to the
17 Hippocratic Corpus of Ancient Greece. The idea that lactic acid bacteria (LAB) fermenting milk are
18 responsible for enhancing health and delaying the human aging was first proposed by the Russian
19 scientist Elie Metchnikoff more than a century ago (Mackowiak, 2013). In the past years, research
20 has documented a wide range of health benefits exerted by dairy LAB, especially immune and
21 metabolic ones, and it is now focusing to decipher the microbial mechanisms underpinning these
22 health-promoting effects (Reid, 2015).

23 Some beneficial effects exerted by LAB are due to the generation of secondary metabolites
24 with health-promoting properties. The most important biogenic compounds in fermented milk are
25 the bioactive peptides released from caseins via the LAB proteolytic system. Biological activities
26 associated with such peptides include immunomodulatory, antibacterial, anti-hypertensive,
27 antioxidant, mineral binding, and opioid-like properties (Brown et al., 2017). In addition, dairy
28 LAB are auxotrophic for many amino acids and efficient casein breakdown is crucial to make LAB
29 competitive as dairy starters (S-LAB), as well as suitable to survive in ripened cheeses as non-
30 starter LAB (NS-LAB) (Kunji, Mierau, Hagting, Poolman, & Konings, 1996). The amino acid
31 release also contributes to the aroma compound formation during cheese ripening and impacts
32 sensorial properties and consumer's acceptance of dairy foods (McSweeney & Sousa, 2000).

33 Cell envelope proteinases (CEPs) are large multi-domain proteins anchored to the cell wall
34 that catalyse the first step of hydrolysis of milk caseins into peptides. Different transport systems
35 then internalize these peptides into the cell, where they are further hydrolysed by numerous
36 intracellular peptidases (Savijoki, Ingmer, & Varmanen, 2006). Six different types of CEPs have
37 been described in several LAB species: PrtB from *Lactobacillus delbrueckii* subsp. *bulgaricus*
38 (Laloi, Atlan, Blanc, Gilbert, & Portalier, 1991); PrtH from *Lactobacillus helveticus* (Genay, Sadat,
39 Gagnaire & Lortal, 2009); PrtL from *Lactobacillus delbrueckii* subsp. *lactis* (Villegas, Brown,

40 Savoy de Giori, & Hebert, 2015); PrtP from *Lactococcus lactis* (Kok, Leenhouts, Haandrikman,
41 Ledeboer, & Venema, 1988), *Lactobacillus paracasei* (Holck & Naes, 1992), *Lactobacillus casei*
42 (Fernández de Palencia, Peláez, Romero, & Martín-Hernández, 1997; Kojic, Fira, Banina, &
43 Topisirovic, 1991), *Lactobacillus rhamnosus* (Guo et al., 2016) and *Lactobacillus plantarum*
44 (Strahinic, Kojic, Tolinacki, Fira, & Topisirovic 2010); PrtR from *Lactobacillus rhamnosus* (Pastar
45 et al., 2003); and PrtS from *Streptococcus thermophilus* (Siezen, 1999). These proteinases vary in
46 substrate specificity, domain composition and cell wall anchoring, but all of them belong to the so-
47 called subtilase family as they contain the catalytic serine protease domain showing sequence
48 homology to the active site of subtilases (Savijoki et al., 2006; Sadat-Mekmene, Genay, Atlan,
49 Lortal, & Gagnaire, 2011a). Most frequently, LAB possess only one CEP, but the presence of two
50 CEPs has been described in lactobacilli (Sadat-Mekmene et al., 2011b).

51 Much of the current knowledge on LAB proteolytic system comes from studies on S-LAB
52 species, such as *Lc. lactis*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. helveticus* and only few works
53 has been done to elucidate the role of NS-LAB. Recently, the NS-LAB species *Lb. paracasei*, *Lb.*
54 *casei* and *Lb. rhamnosus* were proven to generate bioactive casein-derived peptides during milk
55 fermentation (Guo et al., 2016; Solieri, Rutella, & Tagliazucchi, 2015). *Lb. casei/Lb. paracasei*
56 *PrtP*-encoded CEP was also demonstrated to degrade pro-inflammatory chemokines associated to
57 inflammatory bowel diseases (Hormannspurger, von Schillde, & Haller, 2013). Consequently, there
58 is an increasing interest to study NS-LAB proteases responsible for the release of bioactive peptides
59 (Lozo et al., 2011).

60 In our previous work, we demonstrated that two mesophilic NS-LAB strains isolated from
61 Parmigiano Reggiano ripened cheese, namely *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331,
62 exhibit safety and technological performance compatible with probiotic properties (Solieri, Bianchi,
63 Mottolese, Lemmetti, & Giudici, 2014). They also release the angiotensin-I-converting enzyme
64 (ACE)-inhibiting peptides Valine-Proline-Proline (VPP) and Isoleucine-Proline-Proline (IPP) from
65 caseins at doses that may exert antihypertensive effects *in vivo* (Solieri et al., 2015). Despite these

66 multiple interesting properties, the activity and specificity of CEPs from strains PRA205 and
67 PRA331 remain unknown, as well as their potential to release additional milk-derived peptides
68 other than VPP and IPP. The aim of this work was to fill this gap and to evaluate the pattern of
69 casein breakdown by PRA205 and PRA331 whole cells CEP activities through a peptidomic
70 approach.

71 **2. Materials and Methods**

72 *2.1 Microorganisms, media and growth conditions*

73 *Lactobacillus casei* PRA205 and *Lb. rhamnosus* PRA331 were isolated from ripened
74 Parmigiano Reggiano cheese (Solieri, Bianchi, & Giudici, 2012) and deposited in Unimore
75 Microbial Culture Collection (www.umcc.unimore.it) for long-term preservation. The cultures were
76 activated from their frozen forms (stored in MRS medium supplemented with 25% (v/v) glycerol at
77 -80°C) by transferring them in MRS broth and incubating at 37°C for 24h under anaerobic
78 conditions. After two rounds of growth on the same medium, strains were routinely maintained on
79 MRS medium supplemented with 7% (w/v) agar at 4°C for the duration of the experiments.

80 *2.2 Inoculum preparation and milk fermentation*

81 Milk fermentation trials were carried out in triplicate as follows. Single-colony cultures were
82 inoculated in MRS broth for 24h at 37°C . Cells were washed twice with 50 mmol L^{-1} Tris-HCl
83 buffer (pH 6.5), re-suspended in 10% (w/w) skimmed milk and used as pre-cultures (2% v/v) to
84 inoculate milk batches prepared with 50 mL of ultra-high temperature-treated (UHT) skimmed
85 bovine milk. Fermentation was carried out for 72h at 37°C at 10 rpm. pH values were determined
86 over time as previously reported (Solieri et al., 2015). At the end of the fermentation (pH values \leq
87 4.0 for at least two consecutive measurements), samples were taken to estimate milk protein
88 hydrolysis, ACE-inhibitory and radical scavenging activities as reported in section 2.4.

89 *2.3 Cell viability assay*

90 PRA205 and PRA331 cells were harvested by centrifugation after 24, 48 and 72h of milk
91 fermentation, twice washed with physiological solution ($9 \text{ g L}^{-1} \text{ NaCl}$) and re-suspended at the final
92 concentration of 10^7 CFU mL^{-1} , according to the correlation curves between $\text{OD}_{600\text{nm}}$ and CFU
93 values previously established for every strain (Rutella, Tagliazucchi, & Solieri, 2016). Bacterial
94 suspensions were stained with LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen) and
95 live/dead cell ratio was measured according to manufacture instructions. Fluorescence intensity was
96 measured with a Jasco FP-6200 spectrofluorometer (Jasco, Orlando FL, U.S.A.).

97 *2.4 Determination of milk protein hydrolysis, radical scavenging and angiotensin I-converting*
98 *enzyme (ACE)-inhibitory activities*

99 Milk protein hydrolysis and radical scavenging activity were determined on the TCA-
100 soluble supernatants (peptidic fractions) obtained by treating fermented milk with 1% (w/v) TCA
101 followed by a centrifugation at 10,000g for 20 min (4°C). In particular, milk protein hydrolysis was
102 determined by measuring the amounts of released amino groups using the 2,4,6-
103 trinitrobenzenesulfonic acid (TNBS) assay (Adler-Nissen, 1979). Briefly, 50 μL of appropriately
104 diluted peptidic fractions were mixed with 400 μL of sodium phosphate buffer (0.1 mmol L^{-1} ; pH
105 8.2) and 400 μL of 0.1% TNBS solution (prepared in the same sodium phosphate buffer). After 60
106 min of incubation at 50°C , the reactions were stopped by adding 800 μL of $\text{HCl } 0.1 \text{ mmol L}^{-1}$. The
107 absorbance values at 340 nm were read using a Jasco V-550 UV/Vis spectrophotometer (Jasco,
108 Orlando, FL, USA.). A calibration curve was prepared using leucine as standard (range $0.1\text{-}2.0$
109 mmol L^{-1}) and the results were expressed as mmol L^{-1} of leucine equivalents.

110 The antioxidant activity of the peptidic fractions was measured as radical scavenging
111 activity using the ABTS radical cation decolourization assay (Re et al., 1999) and expressed as mg
112 L^{-1} of Trolox.

113 ACE-inhibitory (ACEi) activity was determined according to Ronca-Testoni (1983) on the
114 ultra-filtrated fraction obtained from fermented milk as previously reported (Solieri et al., 2015).
115 The tripeptide 2-furanacryloyl–phenylalanylglycylglycine (FAPGG) was used as substrate assay
116 and the ACEi activity was calculated as percent of inhibition (ACEi%).

117 Three analytical replicates were run for each sample collected at the end of each
118 fermentation trial (carried out in triplicate) in all the assays.

119 *2.5 Determination of peptides with nanoflow LC-ESI-QTOF MS analysis*

120 Peptidomic analysis was performed by injecting the TCA-soluble supernatant of fermented
121 milk on a 1200 Series Liquid Chromatographic two-dimensional system coupled to a 6520
122 Accurate-Mass QTOF LC/MS via a Chip Cube Interface (Agilent Technologies, Santa Clara, CA,
123 USA) as described in Tagliazucchi, Helal, Verzelloni, Bellesia, & Conte (2016). Chromatographic
124 separation was performed on a ProtID-Chip-43(II) including a 4 mm 40 nL enrichment column and
125 a 43 mm × 75 µm analytical column, both packed with a Zorbax 300SB 5 µm C18 phase (Agilent
126 Technologies). The mobile phase consisted of (A) H₂O/acetonitrile/formic acid (96.9:3:0.1, v/v/v)
127 and (B) acetonitrile/H₂O/formic acid (94.9:5:0.1, v/v/v). The sample (2 µL) was loaded onto the
128 Chip enrichment column at a flow rate of 4 µL min⁻¹ with a mobile phase consisting of 100% A
129 using a G1376A capillary pump. A flush volume of 2 µL and a flush-out factor of 5 were used.
130 After valve switching, a gradient elution was performed throughout the enrichment and analytical
131 columns at 500 nL min⁻¹ using a G2226A nano pump. The gradient started at 0% B for 1 min, and
132 then linearly ramped up to 90% B in 70 min. The mobile phase composition was maintained at 90%
133 B for 15 min in order to wash both enrichment and analytical columns. The mass spectrometer was
134 tuned and calibrated according to the manufacturer's instructions in extended dynamic range (2
135 GHz) mode as reported by Dei Più et al. (2014).

136 For peptide identification, MS/MS spectra were converted to .mgf files and were then
137 searched against the Swiss-Prot database using Protein Prospector (<http://prospector.ucsf.edu>) and

138 MASCOT (Matrix Science, Boston, MA, USA) protein identification softwares. The following
139 parameters were considered: enzyme, none; peptide mass tolerance, ± 40 ppm; fragment mass
140 tolerance, ± 0.12 Da; variable modification, oxidation (M) and phosphorylation (ST); maximal
141 number of post-translational modifications permitted in a single peptide, 4. We considered only
142 peptides with a best expected value lower than 0.05 that corresponded to $P < 0.01$. The assignment
143 process was complemented and validated by the manual inspection of MS/MS spectra. Three
144 replicates for each fermentation trial was injected in the mass spectrometer and only the peptides
145 present in at least two replicates were considered significant and included in the analysis.

146 *2.6 Identification of bioactive peptides*

147 The identified peptides in milk samples were investigated for literature-identified bioactive peptides
148 using the BIOPEP database and the Milk Bioactive Peptide Database (MBPDB) (Minkiewicz,
149 Dziuba, Iwaniak, Dziuba, & Darewicz, 2008; Nielsen, Beverly, Qu, & Dallas, 2017). Only peptides
150 with 100% homology to known functional peptides were considered as bioactive peptides.

151 The relative amount of the bioactive peptides was estimated by integrating the area under the peak
152 (AUP). AUP was measured from the extracted ion chromatograms (EIC) obtained for each peptide
153 and normalized to the peptide content of milk hydrolysates. The peptide content was determined at
154 the end of the fermentation trials by using the TNBS method as described in section 2.4 and
155 expressing the results as mg of leucine equivalent mL^{-1}

156 .

157 *2.7 Calculation of the cleavage specificity*

158 The cleavage probability and positive or negative influence on the cleavage of an amino acid
159 in the P1 and P1' subsites were calculated according to Keyl (1992).

160 The subsite nomenclature was according to Schechter & Berger (1967) where the amino
161 acid residues are designated as P1 in the N-terminal direction (on left of the sequence) and P1' in the
162 C-terminal direction (on right of the sequence) from the cleaved bond. The subsite P1 interacts with

163 the subsite S1 in the enzyme active site, whereas the subsite P1' interact with the subsite S1' in the
164 enzyme active site. Therefore, the peptidic bond cleaved by the protease was defined as the P1-P1'
165 bond. We quantitatively analysed the influence of specific amino acid residues in position P1 or
166 P1' on the CEP cleavage probability.

167 If the amino acid residue *A* is in the position *n* (P1 or P1' subsite), the cleavage probability
168 of the P1-P1' bond will be:

169

$$172 \quad \%P_n = \frac{\text{total amino acid } A \text{ cleaved in position } n}{\text{total amino acid } A \text{ in proteins}} \times 100$$

170

171 and in consequence the mean cleavage probability:

$$174 \quad \%P_n = \sum_{\# = 1}^{20} \frac{\%P_n}{20}$$

173

175 The coefficient *Kn* was used to quantify the positive or negative influence of an amino acid
176 residue *A* in the P1 and P1' subsites:

$$177 \quad Kn = \frac{\%P_n}{\%P_n} - 1$$

178 *Kn* values >0 indicated a positive influence of the amino acid *A* in the specific subsite on the
179 cleavage of the P1-P1' bond, whereas *Kn* values <0 suggested a negative effect on the cleavage.

180 2.8 Statistical analysis

181 All data are presented as mean ± standard deviation (SD) for three replicates. The Student's
182 t-test was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The
183 differences were considered significant with *P* <0.05. Venn diagrams were drawn using the online
184 tool VENNY 2.1.0 (Oliveros, 2015).

185 3. Results and Discussion

186 3.1 Characterization of fermented milk

187 Analysis of CEP activities on purified caseins could tend to overestimate the true
188 caseinolytic capability of whole cells towards casein micelles in milk (Sadat-Mekmen et al., 2011b).
189 The use of purified CEPs instead whole-cell anchored CEPs may also modify the specificity of the
190 proteinase towards caseins (Fernández de Palencia et al., 1997). Therefore, we decided to evaluate
191 the CEP activities of whole cells of *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 towards the
192 caseins in UHT milk.

193 Milk samples were inoculated with standardized amounts of PRA205 and PRA331 single
194 cultures without any pre-adaptation step. After 72h of incubation, pH values were 4.00 in both sets
195 of samples and remained stable over time. At the end of fermentation, strain PRA205 showed value
196 of leucine equivalents of $10.93 \pm 0.93 \text{ mmol L}^{-1}$, whereas PRA331 of $6.40 \pm 0.92 \text{ mmol L}^{-1}$. These
197 data agree with earlier results showing that PRA205 is more proteolytic than PRA331 towards milk
198 caseins (Solieri et al., 2015). *Lb. casei* PRA205 produced milk hydrolysates with ACEi activity
199 higher than that exhibited by milk hydrolysates with *Lb. rhamnosus* PRA331 (75.8 ± 3.2 vs $68.5 \pm$
200 2.6 ACEi%). Similarly, antioxidant activity was slightly higher in hydrolysates by strain PRA205
201 than the hydrolysates by strain PRA331 (249.12 ± 15.10 vs $202.57 \pm 18.66 \text{ mg L}^{-1}$ of trolox,
202 respectively).

203 The level of bacterial lysis during milk fermentation was monitored to exclude that the
204 peptides could be generated by intracellular peptidases released into the hydrolysates. Cell viability
205 was estimated app. 100% for both PRA205 and PRA331 after 24 and 48h of incubation (data not
206 showed). Interestingly, at the end of milk fermentation the percentage of viable cells was $90.49 \pm$
207 0.74% and $94.59 \pm 5.70\%$ for PRA205 and PRA331, respectively. These data indicated that no
208 significant lysis occurred during milk fermentation and supported that the observed casein

209 proteolysis was mainly due to the action of CEPs anchored on the whole cells rather than
210 intracellular proteinases or peptidases.

211 3.2. Peptidomic analysis of milk fermented with *Lactobacillus casei* PRA205 and *Lactobacillus* 212 *rhamnosus* PRA331

213 Mass spectrometry analysis was used to identify the full set of peptides present in milk
214 hydrolysates by the selected strains. A total of 331 milk peptides were released by the CEPs
215 activities of PRA205 and PRA331 whole cells. In particular, 178 peptides were identified in
216 PRA205 samples (see supplementary online **Tables S1-S4** and **Figures S1-S4**) and 153 peptides in
217 PRA331 samples (see supplementary online **Tables S5-S8** and **Figures S1-S4**).

218 The analysis of the identified sequences according to their protein of origin showed that the
219 main identified peptides were derived from β -casein, which was the preferred substrate over α S1-,
220 κ - and α S2-caseins. The β -casein-derived peptides were 59.0 and 60.1% of the total identified
221 peptides in PRA205 and PRA331 samples, respectively, followed by α S1-casein-derived peptides
222 (18.5 and 19.0% of the total identified peptides in PRA205 and PRA331 samples, respectively) and
223 κ -casein-derived peptides (16.3 and 15.0% of the total identified peptides in PRA205 and PRA331
224 samples, respectively). PRA205 and PRA331 CEPs poorly hydrolysed α S2-casein, resulting in only
225 11 (corresponding to the 6.2% of total identified peptides) and 8 peptides (corresponding to the
226 5.9% of total identified peptides), respectively. As expected, no significant proteolysis of whey
227 proteins was observed for both the strains. The Venn diagram (**Figure 1**) showed that 24.6 and
228 14.0% of peptides were specific for PRA205 and PRA331 milk hydrolysates, respectively. The
229 majority of the identified peptides were found in both the milk hydrolysates, suggesting that
230 PRA205 and PRA331 share a similar caseinolytic pattern.

231 CEPs are classified on the basis on their caseinolytic specificity (Kunji et al. 1996).
232 Typically, two CEPs have been identified: a P_I-type, which preferentially hydrolyses β -casein, and a
233 P_{III}-type, which acts on α S1-, β - and κ -caseins equally well (Pritchard & Coolbear, 1993; Visser,

234 Exterkate, Slangen & de Veer 1986). A third group, termed P_I/P_{III}-type has been described to
235 classify intermediate proteases, capable to cleave β -casein like the P_I-type and, to a lesser extent, α -
236 and κ -caseins (Exterkate, Alting, & Bruinenberg, 1993). Both *Lb. casei* PRA205 and *Lb. rhamnosus*
237 PRA331 exhibited a predominant CEP activity towards β -casein, and a lower proteolytic activity
238 towards α - and κ -caseins. Cell viability data allowed us to exclude that intracellular aminopeptidase
239 released by lysed cells may significantly contribute to this pattern of casein breakdown.
240 Furthermore, no extracellular aminopeptidases have been reported for *Lb. casei* and *Lb. rhamnosus*
241 (Christensen, Dudley, Pederson, & Steele, 1999). Overall, these evidences support that the observed
242 CEP activities could be due to the mixed P_I/P_{III}-type proteases. P_I/P_{III}-type proteases have been
243 characterized in lactobacilli (Fernandez de Palencia et al., 1997; Sadat-Mekmene et al., 2011a
244 Villegas et al., 2015) and lactococci (Nikolić, Tolinački, Fira, Golić, & Topisirović, 2009). In
245 particular, like PRA331, *Lb. rhamnosus* BGT10 has PrtR protease suitable to cleave both β - and α -
246 caseins.

247 3.3. Analysis of the α S1-casein (f1-23) cleavage sites

248 CEPs are commonly classified according to their specificities toward the α S1-casein fragment
249 comprising residues from 1 to 23 (Exterkate, 1995). In strains PRA205 and PRA331, CEPs
250 hydrolysed the α S1-casein (f1-23) fragment at the H₈-Q₉, Q₉-G₁₀, Q₁₃-E₁₄, N₁₇-E₁₈ and L₂₁-R₂₂
251 positions, respectively (**Figure 2**). In addition, PRA331 also cleaved at the L₁₆-N₁₇ position. The
252 majority of these cleavage sites are typical of mixed P_I/P_{III}-type CEPs isolated from several
253 lactococci, *S. thermophilus* CNRZ 385, *Lb. delbrueckii* subsp. *lactis* CRL 581 and *Lb. helveticus*
254 L89 (Exterkate, 1995; Fernandez-Espla, Garault, Monnet, & Rul, 2000; Hebert et al., 2008; Kunji et
255 al., 1996). Two additional cleavage sites were found at the P₂-K₃ and E₁₈-N₁₉ positions. The
256 cleavage site E₁₈-N₁₉ has been already reported for the CEP of *Lb. delbrueckii* subsp. *lactis* CRL
257 581 (Hebert et al., 2008), whereas the cleavage site P₂-K₃ has never been identified in any CEPs
258 previously described from lactobacilli. These results collectively suggested that CEPs from *Lb.*

259 *casei* PRA205 and *Lb. rhamnosus* PRA331 could belong to the mixed P_I/P_{III}-type. This result
260 disagrees with the P_I-type CEP previously characterized in *Lb. casei* HN14 (Kojic et al., 1991),
261 while it is consistent with the mixed P_I/P_{III} type CEPs isolated from *Lb. rhamnosus* CGMCC11055
262 and *Lb. casei* subsp. *casei* IFLP 731 (Guo et al., 2016; Fernández de Palencia et al., 1997). Overall,
263 these evidences strongly support the high level of intra- and inter-species variability in protease
264 repertoire exhibited by lactobacilli (Liu, Bayjanov, Renckens, Nauta, & Siezen, 2010). As reported
265 above, the cell viability near to 100% measured at the end of the fermentation trials allowed us to
266 exclude that intracellular peptidase released from lysed cells may contribute to the hydrolysis of the
267 fragment α S1-casein (f1-23). Indeed, as reported by Christensen, Broadbent, & Steele (2003), the
268 presence of cytoplasmic peptidase should results in an almost complete breakdown of the peptide
269 α S1-casein (f1-9).

270

271 3.4. Analysis of the β -casein cleavage site-specificity

272 The cleavage site-specificity of PRA205 and PRA331 CEPs was determined using β -casein
273 as preferred substrate (**Figure 3**). In total, 76 and 72 different cleavage sites were detected in
274 samples hydrolysed by PRA205 and PRA331 whole cells, respectively. They constitute 36.5 and
275 34.6% of all peptide bonds present in β -casein, showing that *Lb. casei* PRA205 and *Lb. rhamnosus*
276 PRA331 CEPs have a very broad substrate specificity. These CEPs have almost the same
277 specificity, as they shared 65% of cleavage sites.

278 Amino acid sequence analysis of the identified peptides revealed that the cleavage sites were
279 not concentrated at the N- or C-terminus, but rather distributed throughout the entire β -casein
280 sequence for both the CEPs activities (**Figure 3**). Most of the proteinases previously described in
281 lactobacilli have been proven to preferentially hydrolyse the C-terminal of β -casein (Lozo et al.,
282 2011). Recently, the PrtP proteinase isolated from *Lb. rhamnosus* CGMCC11055 breakdowns sites
283 distributed along the whole β -casein sequence, like PRA205 and PRA331 CEPs (Guo et al., 2016).

284 Furthermore, we calculated the cleavage probability (% P_n) of the *Lb. casei* PRA205 CEP at
285 the P1 and P1' positions (**Table 1**). This CEP cleaved preferentially when the P1 position was
286 occupied by the hydrophobic amino acids M, L and F or the negatively charged amino acids Q and
287 N primarily, and by the polar un-charged amino acid E to a lesser extent. **Table 1** also shows how
288 the amino acids at the P1' position affected cleavage occurrence. PRA205 CEP exhibited cleavage
289 preference towards the residues S, N, A and H in this position, whereas had a reduced preference
290 for M, D, R and Y. Coefficients Kn were calculated to quantify the influence of different amino acid
291 residues on the P1-P1' cleavage probabilities (**Figure 4**). Amino acids N, M, Q, F and L in the P1
292 position and amino acids S, N, A and H in the P1' position exerted the strongest positive effects on
293 cleavage occurrence. The amino acids E in P1 position and V, M, D, R and Y in P1' position also
294 exerted a positive but weaker effect on cleavage probability. By contrast, G, I, P and D in the P1
295 position and P and I in the P1' position strongly inhibited the cleavage probability. Similarly, a
296 negative effect was also found for the amino acids E and T at the P1' position. Previous works
297 found that CEPs preferentially cleave negatively charged and hydrophobic amino acids (Hebert et
298 al., 2008; Juillard et al., 1995; Lozo et al., 2011; Monnet, Ley, & Gonzalez, 1992). For instance, Q
299 and E at the P1 position positively affect the cleavage by CEP from *Lb. delbrueckii* subsp. *lactis*
300 CRL 581 (Hebert et al., 2008), whereas the occurrence of Q and F at the same position positively
301 affects the cleavage by CEPs from *Lb. rhamnosus* BGT10, *Lb. helveticus* BGRA43 and *Lb.*
302 *paracasei* subsp. *paracasei* BGHN14 (Lozo et al., 2011). In *Lb. casei* PRA205 CEP exhibited a
303 pattern of cleavage site preferences similar to P_I/P_{III}-type CEP described in *Lc. lactis* subsp. *lactis*
304 strain NCDO763 (Monnet, Ley, & Gonzalez, 1992), but different from those described for the P_I-
305 type CEP in *Lc. lactis* subsp. *cremoris* strain Wg2 and for the P_I/P_{III}-type CEP in *Lb. rhamnosus*
306 strain CGMCC11055. In strain Wg2 the residue Y at the P1 position and the residues N and T at the
307 P1' position were preferred (Juillard et al., 1995), whereas in strain CGMCC11055 the residue P
308 was preferred in both P1 and P1' subsites (Guo et al., 2016). By contrast, strain NCDO763 had a
309 CEP activity positively affected by Q and N at the P1 position, and by S and A at the P1' position

310 (Monnet et al., 1992). Additionally, the residue P in both the P1 and P1' positions negatively
311 affected CEP cleavage in NCDO763 (Monnet et al., 1992). Similarly, the presence of a P residue
312 bound to one of the preferred cleaved amino acids prevented CEP from *Lb. casei* PRA205 to cut the
313 peptidic bond. For example, the preferentially cleaved amino acids Q and L formed seven and nine
314 peptidic bonds with the amino acid P, respectively, but no one of these bonds was cleaved by
315 PRA205 CEP (**Figure 3**). Finally, PRA205 CEP activity displayed the following two unique
316 properties: M at the P1 position exerted a strong positive effect on cleavage occurrence, whereas I
317 in both the P1 and P1' positions exerted a strong negative effect. To the best of our knowledge, this
318 cleavage site-specificity pattern has never been described in lactobacilli.

319 As reported in **Table 1** and **Figure 4**, CEP from *Lb. rhamnosus* PRA331 had a profile of
320 cleavage specificity similar to *Lb. casei* PRA205. The main differences were the negative effect
321 exerted by the amino acid H at the P1 position and the lack of the positive effect exerted by A at the
322 P1' position.

323 3.5. Identification of bioactive peptides using functional peptides databases

324 The peptides cleaved by PRA205 and PRA331 CEPs in milk hydrolysates were searched against
325 the general bioactive peptide database BIOPEP (Minkiewicz et al., 2008) and the milk bioactive
326 peptide database MBPDB (Nielsen et al., 2017), in order to find peptides which match sequences to
327 known bioactive peptides. Out of 331 identified peptides, 24 shared 100% homologies with
328 functional peptides previously reported to have various bioactivities (**Table 2**). These bioactive
329 peptides represented 13.5% and 13.7% of the peptides totally released by *Lb. casei* PRA205 and *Lb.*
330 *rhamnosus* PRA331 whole cells, respectively. Twenty-one peptides were commonly released by
331 both the strains, whereas three peptides were uniquely identified in samples hydrolysed by *Lb. casei*
332 PRA205 (**Figure 1**). Nineteen bioactive peptides derived from β -casein, four from α S1-casein and
333 one from α S2-casein, whereas no bioactive peptides were found from κ -casein. (**Table 2**). The three
334 *Lb. casei* PRA205-specific bioactive peptides were the β -casein fragments 192-209

335 (LYQEPVLGPVRGPFPIIV), 58-72 (LVYPPFGPIPNSLPQ) and 8-14 (VPGEIVE). Eighteen
336 peptides were ACE-inhibitors, two had immunomodulatory activity, one showed dipeptidyl-
337 peptidase IV (DPPIV) inhibitory activity and one was an antimicrobial peptide. Two peptides,
338 VYPPFGPIP and YPPFGPIP, were multi-functional bioactive peptides with ACEi, antioxidant
339 and opioid agonist or ACEi, DPPIV-inhibitory and opioid agonist activities, respectively (**Table 2**).
340 Among the peptides with ACEi activity, YPPFGPIP, KVLVPVQ, RPKHPIKHQ and LHLPLP
341 showed *in vivo* antihypertensive activity in spontaneously hypertensive rat (Maeno, Yamamoto, &
342 Takano, 1996; Quirós et al., 2007; Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000). For all the
343 other identified bioactive peptides, the bioactivity was previously demonstrated with *in vitro* assays.

344 The physiological effects of bioactive peptides depend on their capability to arrive at the
345 target organs in an active form (Udenigwe, & Fogliano, 2017). This required resistance to
346 gastrointestinal proteases and brush border membrane peptidases, and absorption through the
347 intestinal epithelium. Usually, P-containing peptides are considered resistant to degradation by
348 digestive proteases. Peptides containing from one to four P residues in their sequences and with, in
349 many cases, P at or near to carboxylic end, were found to survive *in vitro* gastro-intestinal digestion
350 (Tagliazucchi et al., 2016). Among the identified bioactive peptides, seven of them were able to
351 survive *in vitro* gastro-intestinal digestion (Picariello et al., 2015; Tagliazucchi et al., 2016) and
352 were also found in human gastro-intestinal tract (Boutrou et al., 2013), namely DKIHFP,
353 VYPPFGPIP, YPPFGPIP, NIPPLTQTPV, LHLPLP, FVAPFPEVF and VAPFPEVF. The
354 intestinal brush-border membrane and the colonic cells also contain aminopeptidases e specific
355 prolyl peptidases. However, the great quantity of P-rich peptides and the presence of peptides with
356 inhibitory activities (as for example against DPP-IV and intestinal ACE) may slow down the action
357 of prolyl peptidases, protecting the short peptides from hydrolysis and favouring their biological
358 actions. Short peptides (two or three amino acids) are absorbed intact across the brush border
359 membrane by a specific peptide transport system, whereas largest peptides via paracellular and/or

360 transcellular mechanisms (Vermeirssen, Van Camp, & Verstraete, 2004). The peptide LHLPLP
361 showed *in vivo* anti-hypertensive activity in rats, and, after incubation with Caco-2 cells, it was
362 hydrolysed by cellular peptidases to HLPLP prior to transport across the intestinal epithelium
363 (Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008). The penta-peptide HLPLP showed an
364 absolute bioavailability of 5.2% and an absorption half-life of 2.8 min in rats (Sánchez-Rivera et al.,
365 2014). HLPLP was found to be hydrolysed by plasma peptidases in shorter peptides, which retained
366 the anti-hypertensive properties in rats (Quirós et al., 2008; Sánchez-Rivera et al., 2014; Sánchez-
367 Rivera et al., 2016). No data on absorption or pharmacokinetics of the other identified bioactive
368 peptides are available in literature.

369

370 *3.6. Bioactive peptides abundance across PRA205 and PRA331 fermented milk*

371 Each identified bioactive peptide was relatively quantified in the samples by integrating the
372 area under the peak (AUP) from the extracted ion chromatogram. The ionization of specific
373 peptides in mass spectrometry experiments is a major limitation in quantitative analysis with
374 electrospray ionization mass spectrometry (ESI-MS). The relative ionization of individual peptides
375 is dependent on intrinsic and extrinsic factors. The most important extrinsic factor is the so-called
376 “matrix effect” which is caused by the co-elution of matrix components (typically salts, ions, highly
377 polar compounds and carbohydrates) that alter, either suppressing or enhancing, the ionization of
378 the target analyte (Furey, Moriarty, Bane, Kinsella, & Lehane 2013). The intrinsic factors are related
379 to the amino acid sequence of the peptides. Some amino acids, such as basic or hydrophobic amino
380 acids, are ionized more efficiently than the others and gave more intense signal in ESI-MS
381 experiments (Cech, & Enke, 2000). Here, we compared the relative amount (expressed as AUP) of
382 the same peptide in two different hydrolysates coming from the same matrix (fermented milk), thus
383 we can exclude errors related to the extrinsic effect and assume that differences in peak intensity of
384 the same analyte accurately reflects relative differences in its abundance.

385 Among the bioactive peptides detected in both milk hydrolysates, 17 exhibited mean
386 abundances significantly different between *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331
387 ($P<0.05$). In particular, five peptides were more abundant in *Lb. casei* PRA205 milk hydrolysates
388 and twelve in *Lb. rhamnosus* PRA331 milk hydrolysates (**Table 2**). When peptides intensities were
389 summed, the bioactive peptides released by *Lb. rhamnosus* PRA331 whole cells were significantly
390 higher than those released by *Lb. casei* PRA205 ($11.50 \times 10^{10} \pm 0.39 \times 10^{10}$ vs. $8.63 \times 10^{10} \pm 0.37 \times 10^{10}$;
391 $P=0.0007$).

392 **4. Conclusions**

393 Nowadays, there is an increasing interest in developing novel dairy healthy products.
394 Studies on strains *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 may represent a proof-of-concept
395 of the working flowchart to develop novel functional adjunct culture and the subsequent functional
396 delivery food. We isolated proteolytic and stress-resistant strains from a stressful food niche (no
397 sugars available, high salt concentration, low aw), such as Parmigiano Reggiano, and identified
398 them using a rigorous polyphasic identification frame-shift (Solieri et al., 2012). We demonstrated
399 that all of them are safe (sensitive to all tested antibiotics) and some resistant to *in vitro* gastro-
400 intestinal conditions (Solieri et al., 2014). We positively tested their ability to release VPP and IPP
401 both in milk (Solieri et al., 2015) and in yogurt (Rutella et al., 2016), supporting the development of
402 a double functional food, i.e. yogurt enriched in potentially probiotic viable cells and in
403 antihypertensive peptides released by themselves. In this work, we characterized the CEPs that are
404 the first enzymatic activities responsible for these relevant proteolytic features. For this purpose, a
405 cutting-edge peptidomic approach was implemented in order to define the pattern of caseins
406 breakdown by CEP activity from whole cells grown in milk. We demonstrated that CEPs activities
407 of *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 showed two unique features: a new cleavage site
408 (P_2-K_3) on the $\alpha S1$ -casein fragment 1-23 and a novel pattern of β -casein cleavage site-specificity.
409 Through a BIOPEP and MBPDB databases analysis, we also demonstrated that several identified

410 peptides matched the sequences of previously reported bioactive peptides. This information could
411 be relevant, mainly considering the wide heterogeneity in distribution of different proteinase-
412 encoding genes among and within *Lactobacillus* species. Comparative genome analysis showed that
413 lactobacilli strongly differ in the components of their proteolytic systems at strain level (Liu et al.,
414 2010). This strain-specificity accounts for the high phenotypic diversity in caseinolytic activity and
415 in of the resulting released bioactive peptides, as well as makes necessary to deeply characterize
416 each strain selected for functional food applications. However, it is important to note that protein
417 hydrolysis catalysed by proteases is a dynamic process and that, in the present work, bioactive
418 peptides were identified in fermented milk samples at one single time. We cannot exclude that
419 shorter or longer incubation of milk with *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 whole
420 cells may result in a different bioactive peptide profile of the samples. In addition, future
421 biochemical assays with the synthesized peptides are needed to complement the *in silico* evidences
422 collected here.

423 Overall, the results provided in the present work will increase the knowledge about the
424 proteolytic system of two important NS-LAB species, such as *Lb. casei* and *Lb. rhamnosus*, which
425 are poorly studied compared to the best-described lactococci and thermophilic lactobacilli. Finally,
426 since strains PRA205 and PRA331 released several potential bioactive peptides, they could be
427 promising functional starters or adjunct cultures for formulating dairy products with health
428 properties.

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Figure Captions

Figure 1. Venn diagram showing differences between *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 CEPs in patterns of peptides and bioactive peptides cleaved from milk caseins. The complete pattern of peptides identified at the end of the fermentation trials by mass spectrometry can be found in Supplementary on line **Tables S1-S8**. In the preparation of the Venn diagram related to bioactive peptides, only peptides found from the literature to have 100% homology to known functional peptides were reported in the **Figure**. Peptides present in at least two of a triplicate's samples were considered present.

Figure 2. Specificity of CEPs from strains *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 toward α S1-casein fragment 1-23. The cleavage sites are indicated by arrows.

Figure 3. Distribution of the cleavage sites identified in the primary sequences of β -casein by CEPs from *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331. The cleavage sites are indicated by arrows.

Figure 4. Cleavage preference of *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 CEPs towards eighteen amino acids at the P1 and P1' subsites. (A) Influence of the different amino acids in the P1 subsite by CEP from *Lb. casei* PRA205. (B) Influence of the different amino acids in the P1' subsite by CEP from *Lb. casei* PRA205. (C) Influence of the different amino acids in the P1 subsite by CEP from *Lb. rhamnosus* PRA331. (D) Influence of the different amino acids in the P1' subsite by CEP from *Lb. rhamnosus* PRA331. See materials and methods section for the calculation of the coefficient *Kn*. Positive and negative values indicate a positive or negative influence exerted by each residue on the cleavage of the P1-P1' bond, respectively. Please note that the amino acid C is not present in the sequence of β -casein, whereas W was omitted from the analysis since it occurs once in the β -casein sequence.

Table 3. Peptides with previously demonstrated bioactivity identified in the milk hydrolysates by whole cells of *Lactobacillus casei* PRA205 or *Lactobacillus rhamnosus* PRA331.

<i>Sequence</i>	<i>Fragment</i>	<i>Bioactivity</i>	<i>PRA205 relative amount^a (±SD)</i>	<i>PRA331 relative amount^a (±SD)</i>	<i>P-value</i>	<i>Reference</i>
LNVPGEIVE	β-casein f(6-14)	ACEi	1.13x10 ⁹ ± 1.26x10 ⁸	1.30x10 ⁹ ± 4.21x10 ⁷	0.0876	Gobbetti et al., 2000
VPGEIVE	β-casein f(8-14)	DPPIV-inhibitor	4.30x10 ⁸ ± 3.38x10 ⁷	n.d.	/	Nongonierma et al., 2016
DKIHPF	β-casein f(47-52)	ACEi	2.37x10 ¹⁰ ± 3.37x10 ⁹	2.36x10 ¹⁰ ± 6.25x10 ⁸	0.4925	Gobbetti et al., 2000
LVYFPFGPIPNLSLPQ	β-casein f(58-72)	ACE-inhibitor	3.44x10 ⁸ ± 3.89x10 ⁷	n.d.	/	Smacchi et al., 2008
VYFPFGPIPN	β-casein f(59-68)	ACEi Antioxidant Opioid agonist	8.19x10 ⁹ ± 7.04x10 ⁸	1.62x10 ¹⁰ ± 4.17x10 ⁸	0.0004	Eisele et al., 2013
YFPFGPIPN	β-casein f(60-68)	ACEi DPPIV-inhibitor Opioid agonist	3.34x10 ⁹ ± 1.30x10 ⁸	1.03x10 ¹⁰ ± 1.89x10 ⁹	0.0030	Saito et al., 2000
NIPPLTQTPV	β-casein f(73-82)	ACEi	9.51x10 ⁹ ± 5.72x10 ⁸	5.96x10 ⁹ ± 4.64x10 ⁸	0.0027	Gobbetti et al., 2000
NLHLPLP	β-casein f(132-138)	ACEi	1.94x10 ⁹ ± 2.09x10 ⁸	7.81x10 ⁸ ± 1.22x10 ⁸	0.0031	Kohmura et al., 1989
NLHLPLPLL	β-casein f(132-140)	ACEi	8.78x10 ⁸ ± 3.33x10 ⁷	5.92x10 ⁸ ± 1.39x10 ⁷	0.0037	Robert et al., 2004
LHLPLP	β-casein f(133-138)	ACEi	3.40x10 ⁸ ± 3.47x10 ⁷	7.64x10 ⁸ ± 6.41x10 ⁷	0.0072	Kohmura et al., 1989
LHLPLPL	β-casein f(133-139)	ACEi	2.55x10 ⁹ ± 2.85x10 ⁸	3.06x10 ⁹ ± 2.19x10 ⁸	0.0920	Quiros et al., 2007
SQSKVLPVPQ	β-casein f(166-175)	ACEi	3.49x10 ⁸ ± 3.51x10 ⁷	6.14x10 ⁸ ± 7.03x10 ⁷	0.0050	Hayes et al., 2007
SKVLPVPQ	β-casein f(168-175)	ACEi	8.32x10 ⁸ ± 1.07x10 ⁸	1.07x10 ⁹ ± 7.70x10 ⁷	0.0380	Yamamoto et al., 1994
KVLPVPQ	β-casein f(169-175)	ACEi	6.05x10 ⁹ ± 5.74x10 ⁸	1.40x10 ¹⁰ ± 1.76x10 ⁹	0.0022	Maeno et al., 1996
RDMPIQAF	β-casein f(183-190)	ACEi	7.64x10 ⁹ ± 2.57x10 ⁸	1.33x10 ⁹ ± 8.06x10 ⁷	<0.0001	Yamamoto et al., 1994
LYQEPVLGPVRGPFPIIV	β-casein f(192-209)	Immunomodulator	3.19x10 ⁸ ± 4.13x10 ⁷	n.d.		Boutrou et al., 2013
YQEPVLGPVRGPFPIIV	β-casein f(193-209)	Immunomodulator	1.69x10 ⁹ ± 3.86x10 ⁸	1.21x10 ⁹ ± 1.57x10 ⁸	0.1237	Boutrou et al., 2013
QEPVLGPVRGPFPIIV	β-casein f(194-209)	ACEi	7.53x10 ⁹ ± 1.96x10 ⁸	1.16x10 ¹⁰ ± 5.14x10 ⁸	0.0044	Lu et al., 2016
EPVLGPVRGPFPP	β-casein f(195-206)	ACEi	3.79x10 ⁸ ± 3.38x10 ⁷	4.90x10 ⁸ ± 4.07x10 ⁷	0.0219	Hayes et al., 2007
RPKHPIKHQ	αS1-casein f(1-9)	ACEi	7.10x10 ⁹ ± 6.63x10 ⁸	1.73x10 ¹⁰ ± 2.66x10 ⁹	0.0032	Saito et al., 2000
ENLLRF	αS1-casein f(18-24)	ACEi	8.23x10 ⁸ ± 1.76x10 ⁸	1.46x10 ⁹ ± 2.66x10 ⁸	0.0226	Boutrou et al., 2013

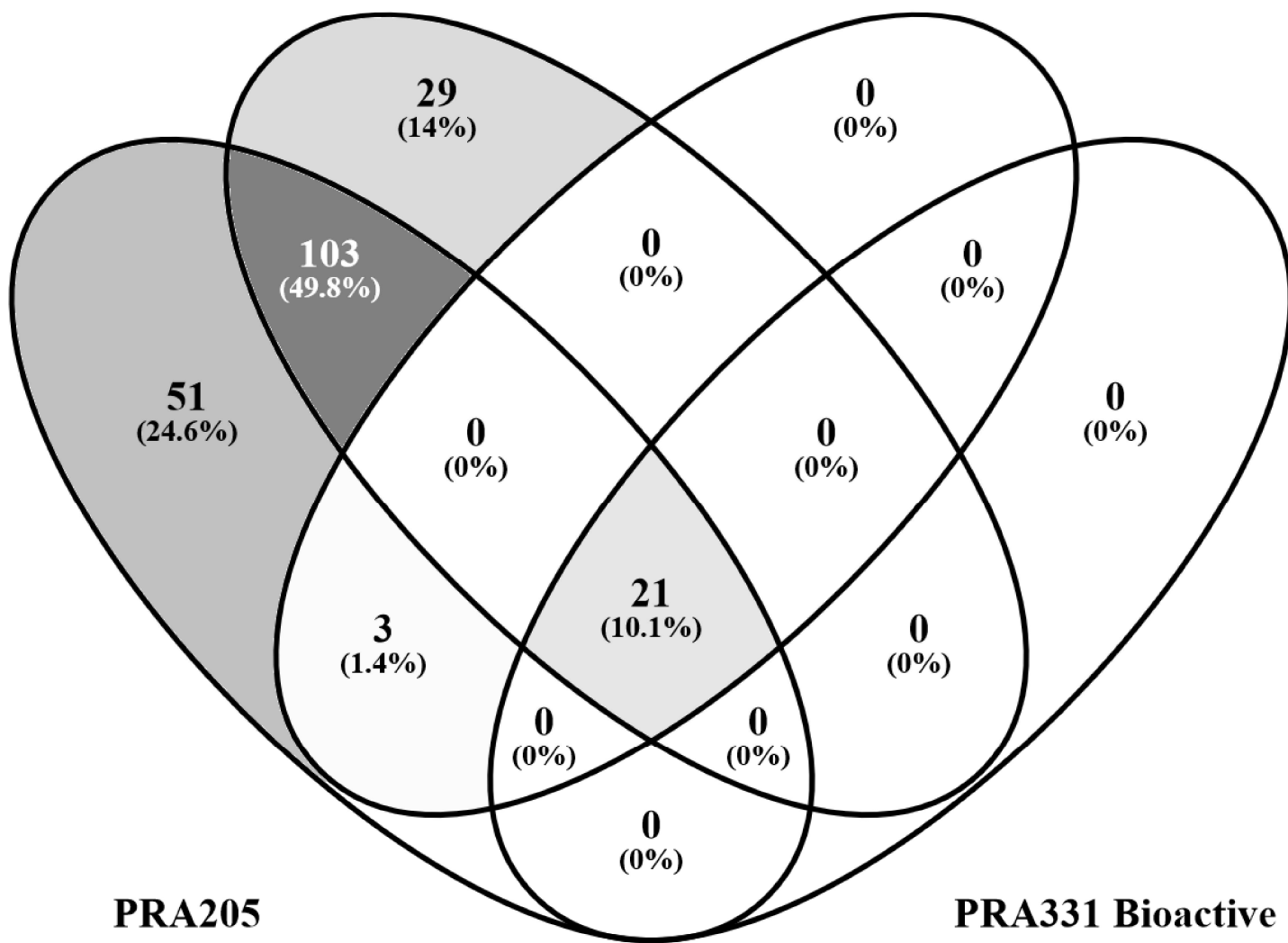
FVAPFPEVF	α S1-casein f(24-32)	ACEi	$7.27 \times 10^8 \pm 1.16 \times 10^8$	$1.80 \times 10^9 \pm 6.93 \times 10^7$	0.0039	Boutrou et al., 2013
VAPFPEVF	α S1-casein f(25-32)	ACEi	$3.17 \times 10^8 \pm 3.39 \times 10^7$	$1.48 \times 10^9 \pm 1.37 \times 10^8$	0.0003	Boutrou et al., 2013
TKVIPYVRYL	α S2-casein f(198-207)	Antimicrobial	$1.62 \times 10^8 \pm 3.75 \times 10^7$	$5.30 \times 10^7 \pm 2.11 \times 10^7$	0.0180	Alvarez- Ordóñez et al., 2013

Abbreviations are: ACEi, angiotensin converting enzyme-inhibitory; DPPIV, dipeptidyl peptidase IV.

^aAmounts were calculated by measuring the area under the peak (AUP) from the extracted ion chromatograms (EIC) obtained for each peptide and AUP values were normalized to the total peptide content of the milk hydrolysates. Values are means \pm standard deviation. Statistically significant differences between PRA205 and PRA331 samples were calculated by Student's t-test ($P < 0.05$).

PRA331

PRA205 Bioactive



PRA205

PRA331 Bioactive

RPKHPIKHQGLPQEVLNENLLRF

PRA205

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↑

↑

↑

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PRA331

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RELEELNVPGEIVESLSSEESITRINKKIEKFQSEE

PRA205 ↑↑↑ ↑ ↑ ↑ ↑ ↑↑↑↑
PRA331 ↑↑ ↑ ↑ ↑ ↑ ↑

QQQTEDELQDKIHPFAQTQSLVYFPFGPIPNLQPQ

PRA205 ↑↑ ↑ ↑↑↑↑ ↑ ↑
PRA331 ↑ ↑↑ ↑↑↑↑ ↑ ↑↑↑

NIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKH

PRA205 ↑↑↑ ↑ ↑↑↑ ↑ ↑ ↑ ↑↑↑↑↑
PRA331 ↑↑ ↑ ↑↑ ↑↑↑ ↑ ↑ ↑↑↑↑

KEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQ

PRA205 ↑↑ ↑↑ ↑ ↑↑↑↑ ↑↑↑↑↑
PRA331 ↑↑ ↑↑ ↑ ↑↑↑↑ ↑↑↑↑↑

SWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVP

PRA205 ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑↑↑↑↑↑
PRA331 ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑↑↑↑↑↑

QKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV

PRA205 ↑ ↑↑ ↑↑↑↑↑↑↑ ↑ ↑↑↑ ↑ ↑↑↑
PRA331 ↑ ↑↑ ↑ ↑↑↑↑↑↑↑ ↑ ↑↑↑ ↑ ↑↑↑

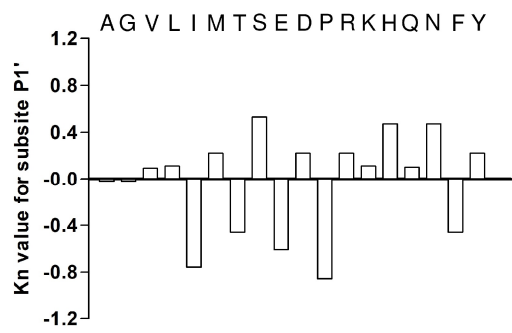
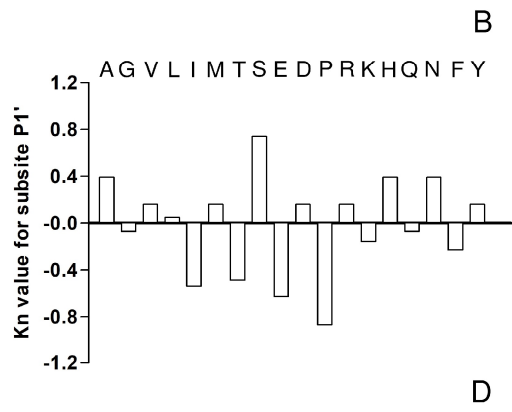
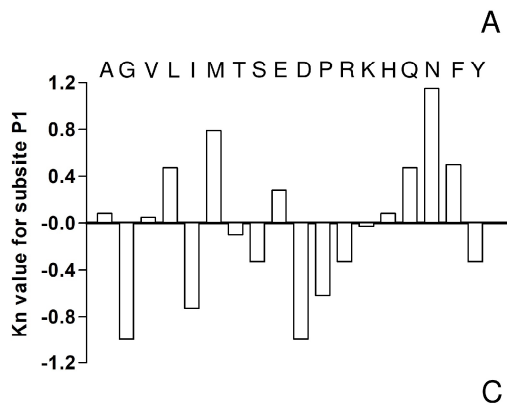


Table 1. Cleavage occurrence and cleavage probability (%P) produced by *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 cell-envelope proteinase on β -casein at different amino acids in the P1 and P1' subsites.

Amino acids ^a	<i>Lb. casei</i> PRA205			<i>Lb. rhamnosus</i> PRA331		
	Number of residues	P1 subsite	P1' subsite	Number of residues	P1 subsite	P1' subsite
		Number of cleaved bond ^b (%P1 ^c)	Number of cleaved bond ^b (%P1 ^c)		Number of cleaved bond ^b (%P1 ^c)	Number of cleaved bond ^b (%P1 ^c)
<i>Aliphatic amino acids</i>						
A	5	2 (40.0)	3 (60.0)	5	2 (40.0)	2 (40.0)
G	5	0 (0)	2 (40.0)	5	0 (0)	2 (40.0)
V	18	7 (38.9)	9 (50.0)	18	5 (27.8)	8 (44.4)
L	22	12 (54.6)	10 (45.5)	22	12 (54.6)	10 (45.5)
I	10	1 (10.0)	2 (20.0)	10	2 (20.0)	1 (10.0)
M	6	4 (66.7)	3 (50.0)	6	4 (66.7)	3 (50.0)
<i>Polar un-charged amino acids</i>						
T	9	3 (33.3)	2 (22.2)	9	3 (33.3)	2 (22.2)
S	16	4 (25.0)	12 (75.0)	16	5 (31.3)	10 (61.5)
E	19	9 (47.4)	3 (15.8)	19	8 (42.1)	3 (15.8)
D	4	0 (0)	2 (50.0)	4	0 (0)	2 (50.0)
P	35	5 (14.3)	2 (5.7)	35	6 (17.1)	2 (5.7)
<i>Positively charged amino acids</i>						
R	4	1 (25.0)	2 (50.0)	4	1 (25.0)	2 (50.0)
K	11	4 (36.4)	4 (36.4)	11	3 (27.3)	5 (45.5)
H	5	2 (40.0)	3 (60.0)	5	1 (20.0)	3 (60.0)
<i>Negatively charged amino acids</i>						
Q	20	11 (55.0)	8 (40.0)	20	10 (50.0)	9 (45.0)
N	5	4 (80.0)	3 (60.0)	5	3 (60.0)	3 (60.0)
<i>Aromatic amino acids</i>						
F	9	5 (55.6)	3 (33.3)	9	5 (55.7)	2 (22.2)
Y	4	1 (25.0)	2 (50.0)	4	1 (25.0)	2 (50.0)

^aOne code letter was used for amino acid nomenclature. The amino acid C is not present in the sequence of β -casein, while the amino acid W was omitted from the analysis since it occurs once in the β -casein sequence.

^bThe cleaved bonds are reported in **Figure 3**.

^cSee materials and methods section for the calculation of the %PI and %PI' cleavage probability.

Table 2. Peptides with previously demonstrated bioactivity identified in the milk hydrolysates by whole cells of *Lactobacillus casei* PRA205 or *Lactobacillus rhamnosus* PRA331.

<i>Sequence</i>	<i>Fragment</i>	<i>Bioactivity</i>	<i>PRA205 relative amount^a (±SD)</i>	<i>PRA331 relative amount^a (±SD)</i>	<i>P-value</i>	<i>Reference</i>
LNVPGEIVE	β-casein f(6-14)	ACEi	1.13x10 ⁹ ± 1.26x10 ⁸	1.30x10 ⁹ ± 4.21x10 ⁷	0.0876	Gobbetti et al., 2000
VPGEIVE	β-casein f(8-14)	DPPIV-inhibitor	4.30x10 ⁸ ± 3.38x10 ⁷	n.d.	/	Nongonierma et al., 2016
DKIHPF	β-casein f(47-52)	ACEi	2.37x10 ¹⁰ ± 3.37x10 ⁹	2.36x10 ¹⁰ ± 6.25x10 ⁸	0.4925	Gobbetti et al., 2000
LVYFPFGPIPNSLPQ	β-casein f(58-72)	ACE-inhibitor	3.44x10 ⁸ ± 3.89x10 ⁷	n.d.	/	Smacchi et al., 2008
VYFPFGPIP	β-casein f(59-68)	ACEi Antioxidant Opioid agonist	8.19x10 ⁹ ± 7.04x10 ⁸	1.62x10 ¹⁰ ± 4.17x10 ⁸	0.0004	Eisele et al., 2013
YFPFGPIP	β-casein f(60-68)	ACEi DPPIV-inhibitor Opioid agonist	3.34x10 ⁹ ± 1.30x10 ⁸	1.03x10 ¹⁰ ± 1.89x10 ⁹	0.0030	Saito et al., 2000
NIPPLTQTPV	β-casein f(73-82)	ACEi	9.51x10 ⁹ ± 5.72x10 ⁸	5.96x10 ⁹ ± 4.64x10 ⁸	0.0027	Gobbetti et al., 2000
NLHLPLP	β-casein f(132-138)	ACEi	1.94x10 ⁹ ± 2.09x10 ⁸	7.81x10 ⁸ ± 1.22x10 ⁸	0.0031	Kohmura et al., 1989
NLHLPLPLL	β-casein f(132-140)	ACEi	8.78x10 ⁸ ± 3.33x10 ⁷	5.92x10 ⁸ ± 1.39x10 ⁷	0.0037	Robert et al., 2004
LHLPLP	β-casein f(133-138)	ACEi	3.40x10 ⁸ ± 3.47x10 ⁷	7.64x10 ⁸ ± 6.41x10 ⁷	0.0072	Kohmura et al., 1989
LHLPLPL	β-casein f(133-139)	ACEi	2.55x10 ⁹ ± 2.85x10 ⁸	3.06x10 ⁹ ± 2.19x10 ⁸	0.0920	Quiros et al., 2007
SQSKVLPVPQ	β-casein f(166-175)	ACEi	3.49x10 ⁸ ± 3.51x10 ⁷	6.14x10 ⁸ ± 7.03x10 ⁷	0.0050	Hayes et al., 2007
SKVLPVPQ	β-casein f(168-175)	ACEi	8.32x10 ⁸ ± 1.07x10 ⁸	1.07x10 ⁹ ± 7.70x10 ⁷	0.0380	Yamamoto et al., 1994
KVLPVPQ	β-casein f(169-175)	ACEi	6.05x10 ⁹ ± 5.74x10 ⁸	1.40x10 ¹⁰ ± 1.76x10 ⁹	0.0022	Maeno et al., 1996
RDMPIQAF	β-casein f(183-190)	ACEi	7.64x10 ⁹ ± 2.57x10 ⁸	1.33x10 ⁹ ± 8.06x10 ⁷	<0.0001	Yamamoto et al., 1994
LYQEPVLGPVRGPFPIIV	β-casein f(192-209)	Immunomodulator	3.19x10 ⁸ ± 4.13x10 ⁷	n.d.		Boutrou et al., 2013
YQEPVLGPVRGPFPIIV	β-casein f(193-209)	Immunomodulator	1.69x10 ⁹ ± 3.86x10 ⁸	1.21x10 ⁹ ± 1.57x10 ⁸	0.1237	Boutrou et al., 2013
QEPVLGPVRGPFPIIV	β-casein f(194-209)	ACEi	7.53x10 ⁹ ± 1.96x10 ⁸	1.16x10 ¹⁰ ± 5.14x10 ⁸	0.0044	Lu et al., 2016
EPVLGPVRGPF	β-casein f(195-206)	ACEi	3.79x10 ⁸ ± 3.38x10 ⁷	4.90x10 ⁸ ± 4.07x10 ⁷	0.0219	Hayes et al., 2007
RPKHPIKHQ	αS1-casein f(1-9)	ACEi	7.10x10 ⁹ ± 6.63x10 ⁸	1.73x10 ¹⁰ ± 2.66x10 ⁹	0.0032	Saito et al., 2000
ENLLRF	αS1-casein f(18-24)	ACEi	8.23x10 ⁸ ± 1.76x10 ⁸	1.46x10 ⁹ ± 2.66x10 ⁸	0.0226	Boutrou et al., 2013

FVAPFPEVF	α S1-casein f(24-32)	ACEi	$7.27 \times 10^8 \pm 1.16 \times 10^8$	$1.80 \times 10^9 \pm 6.93 \times 10^7$	0.0039	Boutrou et al., 2013
VAPFPEVF	α S1-casein f(25-32)	ACEi	$3.17 \times 10^8 \pm 3.39 \times 10^7$	$1.48 \times 10^9 \pm 1.37 \times 10^8$	0.0003	Boutrou et al., 2013
TKVIPYVRYL	α S2-casein f(198-207)	Antimicrobial	$1.62 \times 10^8 \pm 3.75 \times 10^7$	$5.30 \times 10^7 \pm 2.11 \times 10^7$	0.0180	Alvarez- Ordóñez et al., 2013

Abbreviations are: ACEi, angiotensin converting enzyme-inhibitory; DPPIV, dipeptidyl peptidase IV. Only peptides found from the literature to have 100% homology to known functional peptides were reported in the **Table**. The complete list of identified peptides can be found in Supplementary on line **Tables S1-S8**.

^aAmounts were calculated by measuring the area under the peak (AUP) from the extracted ion chromatograms (EIC) obtained for each peptide and AUP values were normalized to the total peptide content of the milk hydrolysates as described in section 2.6. Values are means \pm standard deviation. Statistically significant differences between PRA205 and PRA331 samples were calculated by Student's t-test ($P < 0.05$).

Figure S1. β -casein derived peptides identified in the TCA-soluble supernatant obtained from milk fermented with *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331. The peptides are reported as bars below the corresponding amino acid sequences. Red bars correspond to peptides with angiotensin-converting enzyme (ACE) inhibitory activity. Yellow bars correspond to peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory activity. Green bars correspond to peptides with antioxidant activity. Light blue bars correspond to peptides with anti-microbial activity. Blue bars correspond to peptides with opioid agonist activity. Purple bars correspond to peptides with immunomodulation activity. Peptides with more than one activity are presented with multi-colour bars.

Figure S2. α S1-casein derived peptides identified in the TCA-soluble supernatant obtained from milk fermented with *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331. The peptides are reported as bars below the corresponding amino acid sequences. Red bars correspond to peptides with angiotensin-converting enzyme (ACE) inhibitory activity. Yellow bars correspond to peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory activity. Green bars correspond to peptides with antioxidant activity. Light blue bars correspond to peptides with anti-microbial activity. Blue bars correspond to peptides with opioid agonist activity. Purple bars correspond to peptides with immunomodulation activity. Peptides with more than one activity are presented with multi-colour bars.

Figure S3. α S2-casein derived peptides identified in the TCA-soluble supernatant obtained from milk fermented with *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331. The peptides are reported as bars below the corresponding amino acid sequences. Red bars correspond to peptides with angiotensin-converting enzyme (ACE) inhibitory activity. Yellow bars correspond to peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory activity. Green bars correspond to peptides with antioxidant activity. Light blue bars correspond to peptides with anti-microbial activity. Blue bars correspond to peptides with opioid agonist activity. Purple bars correspond to

peptides with immunomodulation activity. Peptides with more than one activity are presented with multi-colour bars.

Figure S4. κ -casein derived peptides identified in the TCA-soluble supernatant obtained from milk fermented with *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331. The peptides are reported as bars below the corresponding amino acid sequences. Red bars correspond to peptides with angiotensin-converting enzyme (ACE) inhibitory activity. Yellow bars correspond to peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory activity. Green bars correspond to peptides with antioxidant activity. Light blue bars correspond to peptides with anti-microbial activity. Blue bars correspond to peptides with opioid agonist activity. Purple bars correspond to peptides with immunomodulation activity. Peptides with more than one activity are presented with multi-colour bars.

Lactobacillus casei PRA205

RELEELNVPGEIVESLSSEESITRINKKIEKFQSEEQQTEDELQDKIH PFAQTQSLVYPFGPIPNLSLPQNIPP

LTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQ

PHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVVRGPFPIIV

Lactobacillus rhamnosus PRA331

RELEELNVPGEIVESLSSEESITRINKKIEKFQSEEQQTEDELQDKIH PFAQTQSLVYPFGPIPNLSLPQNIPP

LTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQ

PHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVVRGPFPIIV

Lactobacillus casei PRA205

RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQMEAESISSSEEIVPNSV



EQKHIQKEDVPSERYLGYLEQLRLKKYKVPQLEIVPNSAEERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFR



QFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW



Lactobacillus rhamnosus PRA331

RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQMEAESISSSEEIVPNSV



EQKHIQKEDVPSERYLGYLEQLRLKKYKVPQLEIVPNSAEERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFR



QFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW



Lactobacillus casei PRA205

KNTMEHVSSEESIISQETYKQEKNMAINPSKENLCSTFCKEVVRNANEEEYSIGSSSEESAEVATEEVKITVDDK
HYQKALNEINQFYQKFPQYLQYLYQGPIVLNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVF~~TKK~~
TKL~~TEEE~~KNRLN~~FL~~KKISQRYQKFALPQYLKTVYQH~~Q~~KAMKPW~~IQ~~PKTKVIPYVRYL

Lactobacillus rhamnosus PRA331

KNTMEHVSSEESIISQETYKQEKNMAINPSKENLCSTFCKEVVRNANEEEYSIGSSSEESAEVATEEVKITVDDK
HYQKALNEINQFYQKFPQYLQYLYQGPIVLNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVF~~TKK~~
TKL~~TEEE~~KNRLN~~FL~~KKISQRYQKFALPQYLKTVYQH~~Q~~KAMKPW~~IQ~~PKTKVIPYVRYL

Lactobacillus casei PRA205

QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPYAKPAAVRSQAQLQ

WQVLSNTVPAKSCQAQPTT██████████MARH██████████PH██████████PHLS██████████FMAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDS

PEVIESPPEINTVQVTSTAV

Lactobacillus rhamnosus PRA331

QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPYAKPAAVRSQAQLQ

WQVLSNTVPAKSCQAQPTT██████████MARH██████████PH██████████PHLS██████████FMAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDS

PEVIESPPEINTVQVTSTAV

Table S1. β -casein-derived peptides identified in milk fermented with *Lactobacillus casei* PRA205 whole cells^a.

<i>Sequence^b</i>	<i>Observed mass (m/z)^c</i>	<i>Calculated mass^d</i>	<i>Fragment</i>	<i>Bioactivity^e</i>	<i>Reference</i>
LNVPGEIVE	969.5562	968.5179	f(6-14)	ACEi	Gobbetti et al., 2000
NVPGEIVE	856.4430	855.4338	f(7-14)	/	/
VPGEIVE	742.3943	741.3909	f(8-14)	DPPIV-inhibitor	Nongonierma et al., 2016
SITRIN	352.1970	702.4024	f(22-27)	/	/
KKIEKF	396.7429	791.4905	f(28-33)	/	/
KKIEKFQ	460.7709	919.5491	f(28-34)	/	/
KKIEKFQS(phospho)E	608.8120	1215.5900	f(28-36)	/	/
KKIEKFQS(phospho)EE	673.3181	1344.6326	f(28-37)	/	/
IEKFQS(phospho)EE	545.2067	1088.4427	f(30-37)	/	/
DKIHPF	756.3786	755.3966	f(47-52)	ACEi	Gobbetti et al., 2000
DKIHPFAQTQ	592.7785	1183.5986	f(47-56)	/	/
SLVYFPFGPIPN	650.8484	1299.6863	f(57-68)	/	/
SLVYFPFGPIPNSLPQ	863.4501	1724.9138	f(57-72)	/	/
LVYFPFGPIPN	1213.6762	1212.6543	f(58-68)	/	/
LVYFPFGPIPNSLPQ	819.9604	1724.9138	f(58-72)	ACEi	Smacchi et al., 2008
VYFPFGPIPN	1100.5568	1099.5702	f(59-68)	ACEi Antioxidant Opioid agonist	Eisele et al., 2013
VYFPFGPIPNSLPQ	763.4073	1524.7977	f(59-72)	/	/
YFPFGPIPN	1001.5291	1000.5018	f(60-68)	ACEi DPPIV-inhibitor Opioid agonist	Saito et al., 2000
SLPQNIPPL	978.5771	977.5546	f(69-77)	/	/
SLPQNIPPLTQTPVVVPPFLQPEVM(ox)	919.8153	2755.4823	f(69-93)	/	/
NIPPLTQTPV	1079.6176	1078.6023	f(73-82)	ACEi	Gobbetti et al., 2000

NIPPLTQTPVVVPPF	809.9563	1617.9131	f(73-87)	/	/
NIPPLTQTPVVVPPFLQPEVM	772.7640	2315.2599	f(73-93)	/	/
TQTPV	545.2884	544.2857	f(78-82)	/	/
TQTPVVVPPF	1084.5754	1083.5965	f(78-87)	/	/
TQTPVVVPPFLQPE	776.4502	1550.8345	f(78-91)	/	/
TQTPVVVPPFLQPEVM	891.4896	1780.9434	f(78-93)	/	/
TQTPVVVPPFLQPEVMGV	969.5306	1937.0333	f(78-95)	/	/
TQTPVVVPPFLQPEVMGVSKVKEAMAP	960.5079	2878.5337	f(78-104)	/	/
QTPVVVPPFLQPEVM	840.9568	1679.8957	f(79-93)	/	/
PVVVPPFLQPEVM	726.4071	1450.7894	f(81-93)	/	/
VVPPFLQPE	1025.6040	1024.5593	f(83-91)	/	/
VVPPFLQPEVM	1255.6987	1254.6682	f(83-93)	/	/
VPPFLQPE	463.7369	925.4909	f(84-91)	/	/
VPPFLQPEVM	578.7970	1155.5968	f(84-93)	/	/
PPFLQPE	827.4361	826.4225	f(85-91)	/	/
LQPEVM	716.3580	715.3575	f(88-93)	/	/
AMAPKHKEMPFPKYPVEPF	748.7340	2243.1271	f(101-119)	/	/
MAPKHKEMPFPKYPVEPF	725.0321	2172.0900	f(102-119)	/	/
APKHKEMPFPKYPVEPF	681.3579	2041.0495	f(103-119)	/	/
APKHKEMPFPKYPVEPFOTESQ	622.5635	2486.2304	f(103-123)	/	/
KHKEMPFPKYPVEPF	625.3218	1872.9596	f(105-119)	/	/
HKEMPFPKYPVEPF	582.6372	1744.8647	f(106-119)	/	/
HKEMPFPKYPVEPFOTESQ	730.9973	2190.0456	f(106-123)	/	/
EMPFPKYPVEP	667.3054	1332.6424	f(108-118)	/	/
EMPFPKYPVEPF	740.8620	1479.7108	f(108-119)	/	/
MPFPKYPVEP	602.8135	1203.5998	f(109-118)	/	/

MPFPKYVPEPF	676.3415	1350.6682	f(109-119)	/	/
NLHLPLP	402.2601	801.4701	f(132-138)	ACEi	Kohmura et al., 1989
NLHLPLPL	916.5616	915.5542	f(132-139)	/	/
NLHLPLPLL	515.3168	1028.6382	f(132-140)	ACEi	Robert et al., 2004
NLHLPLPLLQ	579.3496	1156.6968	f(132-141)	/	/
NLHLPLPLLQS	622.8851	1243.7288	f(132-142)	/	/
NLHLPLPLLQSW	715.9295	1429.8082	f(132-143)	/	/
LHLPLP	345.2209	688.4272	f(133-138)	ACEi	Kohmura et al., 1989
LHLPLPL	401.7546	801.5112	f(133-139)	ACEi	Quiros et al., 2007
LHLPLPLLQ	1043.6493	1042.6539	f(133-141)	/	/
LHLPLPLLQS	565.8369	1129.6859	f(133-142)	/	/
LHLPLPLLQSW	658.9507	1315.7652	f(133-143)	/	/
HLPLPLLQ	465.7873	929.5698	f(134-141)	/	/
HLPLPLLQSW	602.3328	1202.6812	f(134-143)	/	/
LPLPLLQ	793.5148	792.5109	f(135-141)	/	/
LPLPLLQSW	1066.6182	1065.6223	f(135-143)	/	/
WMHQPHQLPPT	490.2347	1467.7081	f(143-154)	/	/
WMHQPHQLPPTVM	566.9374	1697.8170	f(143-156)	/	/
MHQPHQLPPT	641.8116	1281.6288	f(144-154)	/	/
MHQPHQLPPTVM	756.8776	1511.7377	f(144-156)	/	/
MHQPHQLPPTVMFPPQ	661.3254	1982.3793	f(144-160)	/	/
HQPHQLPPT	576.2953	1150.5883	f(145-154)	/	/
HQPHQLPPTVM	691.3551	1370.6972	f(145-156)	/	/
HQPHQLPPTVMFPPQ	617.6508	1849.9298	f(145-160)	/	/
QPHQLPPTVM	622.8084	1243.6383	f(146-156)	/	/
VMFPPQ	359.6717	717.3520	f(155-160)	/	/

VMFPPQSVL	1017.5629	1016.5365	f(155-163)	/	/
FPPQSVL	787.4301	786.4276	f(157-163)	/	/
SQSKVLPVPQ	541.8009	1071.6132	f(166-175)	ACEi	Hayes et al., 2007
QSKVLPVPQ	995.5916	994.5811	f(167-175)	/	/
QSKVLPVPQKAVPYPQR	484.5272	1934.1102	f(167-182)	/	/
SKVLPVPQ	434.2542	866.5226	f(168-175)	ACEi	Yamamoto et al., 1994
KVLPVPQ	390.7414	779.4905	f(169-175)	ACEi	Maeno et al., 1996
VLPVPQ	652.3973	651.3956	f(170-175)	/	/
KAVPYPQ	401.7171	801.4385	f(176-182)	/	/
KAVPYPQR	479.7656	957.5396	f(176-183)	/	/
RDMPIQA	415.7139	829.4116	f(183-189)	/	/
RDMPIQAF	489.2411	976.4800	f(183-190)	ACEi	Yamamoto et al., 1994
RDMPIQAFL	602.3328	1202.6481	f(183-192)	/	/
LYQEPVL	861.4568	860.4644	f(192-198)	/	/
LYQEPVLGPVRGPF	834.9509	1667.9035	f(192-206)	/	/
LYQEPVLGPVRGPFPIIV	997.5960	1993.1401	f(192-209)	Immunomodulator	Boutrou et al., 2013
YQEPVL	748.3554	747.3803	f(193-198)	/	/
YQEPVLGPVRGPF	778.4137	1554.8195	f(193-206)	/	/
YQEPVLGPVRGPFPIIV	941.0462	1880.0560	f(193-209)	Immunomodulator	Boutrou et al., 2013
QEPVL	585.3251	584.3170	f(194-198)	/	/
QEPVLGPVRGPF	696.8883	1391.7561	f(194-206)	/	/
QEPVLGPVRGPFPII	809.9563	1617.9243	f(194-208)	/	/
QEPVLGPVRGPFPIIV	859.4987	1716.9927	f(194-209)	ACEi	Lu et al., 2016
EPVLGPVRGPF	632.8584	1263.6976	f(195-206)	ACEi	Hayes et al., 2007
EPVLGPVRGPFPIIV	795.4756	1588.9341	f(195-209)	/	/
VLGPVRGPFPIIV	682.4140	1362.8388	f(197-209)	/	/

LGPVRGPFPIIV	632.8902	1263.7703	f(198-209)	/	/
GPVRGPFPP	413.7223	825.4497	f(199-206)	/	/
GPVRGPFPII	526.8104	1051.6179	f(199-208)	/	/
GPVRGPFPIIV	576.3457	1150.6863	f(199-209)	/	/
RGPFPIIV	449.7709	897.5436	f(202-209)	/	/

^aAbbreviations are: ACEi, angiotensin converting enzyme-inhibitory; DPPiV, dipeptidyl peptidase IV.

^bOne code letter was used for amino acid nomenclature.

^cThe observed mass is reported as [M+nH]ⁿ⁺.

^dThe calculated mass is in Da.

^ePotential bioactivities were retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

Table S2. α S1-casein-derived peptides identified in milk fermented with *Lactobacillus casei* PRA205 whole cells^a.

<i>Sequence^b</i>	<i>Observed mass (m/z)^c</i>	<i>Calculated mass^d</i>	<i>Fragment</i>	<i>Bioactivity^e</i>	<i>Reference</i>
RPKHPIKH	338.2150	1011.6090	f(1-8)	/	/
RPKHPIKHQ	570.8322	1139.6676	f(1-9)	ACEi	Saito et al., 2000
RPKHPIKHQGLPQ	512.6430	1534.8844	f(1-13)	/	/
RPKHPIKHQGLPQEVLN	498.5285	1990.1224	f(1-17)	/	/
KHPIKHQ	296.4995	886.5137	f(3-9)	/	/
GLPQEVLNE	499.7432	997.5080	f(10-18)	/	/
ENLLRF	396.2126	790.4337	f(18-24)	ACEi	Boutrou et al., 2013
FVAPFPE	806.3771	805.4010	f(24-30)	/	/
FVAPFPEVF	1052.5566	1051.5379	f(24-32)	ACEi	Boutrou et al., 2013
FVAPFPEVFGKE	683.8712	1365.6969	f(24-35)	/	/
VAPFPE	659.3117	658.3326	f(25-30)	/	/
VAPFPEVF	453.2311	904.4695	f(25-32)	ACEi	Boutrou et al., 2013
VAPFPEVFGK	545.7962	1089.5859	f(25-34)	/	/
VAPFPEVFGKE	610.2613	1218.6285	f(25-35)	/	/
VFGKEKV	403.7262	805.4698	f(31-37)	/	/
VFGKEKVN	307.4989	919.5127	f(31-38)	/	/
VFGKEKVNEL	581.8127	1161.6394	f(31-40)	/	/
S(phospho)VEQKHIQ	524.7428	1047.4750	f(75-82)	/	/
RLKKYKVPQ	387.2312	1158.7237	f(100-108)	/	/
KKYKVPQ	445.7761	889.5385	f(102-108)	/	/
KYKVPQ	381.7173	761.4436	f(103-108)	/	/
LEIVPN	684.3778	683.3854	f(109-114)	/	/
S(phospho)AEELRH	461.1781	920.3753	f(115-121)	/	/
SMKEGIH	401.1859	800.3851	f(122-128)	/	/

KEGIHAQ	391.7040	781.4082	f(124-130)	/	/
AQQKEPM	416.1894	830.3956	f(139-135)	/	/
QKEPMIGVN	508.2554	1014.5168	f(131-139)	/	/
FSDIPNPIGSE	1175.5582	1174.5506	f(179-189)	/	/
FSDIPNPIGSEN	645.2999	1288.5935	f(179-190)	/	/
FSDIPNPIGSENSE	753.3396	1504.6682	f(179-192)	/	/
FSDIPNPIGSENSEK	817.3947	1632.7631	f(179-193)	/	/
SDIPNPIGSENSE	679.7936	1357.5997	f(180-192)	/	/
DIPNPIGSENSE	636.2773	1270.5677	f(181-192)	/	/

^aAbbreviation is: ACEi, angiotensin converting enzyme-inhibitory.

^bOne code letter was used for amino acid nomenclature.

^cThe observed mass is reported as [M+nH]ⁿ⁺.

^dThe calculated mass is in Da.

^ePotential bioactivities were retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

Table S3. α S2-casein-derived peptides identified in milk fermented with *Lactobacillus casei* PRA205 whole cells.

<i>Sequence^a</i>	<i>Observed mass (m/z)^b</i>	<i>Calculated mass^c</i>	<i>Fragment</i>	<i>Bioactivity^d</i>	<i>Reference</i>
SIIS(phospho)QETYS	574.7641	1147.5162	f(13-21)	/	/
RNAVPITPT	484.7610	967.5451	f(114-122)	/	/
NAVPITPT	812.4495	811.4440	f(115-122)	/	/
NAVPITPTLNRE	662.8478	1323.7146	f(115-126)	/	/
AVPITPT	698.4023	697.4010	f(116-122)	/	/
AVPITPTLNRE	605.8537	1209.6717	f(116-126)	/	/
LNREQLS(phospho)TS(phospho)EE	733.2800	1464.5534	f(123-133)	/	/
NSKKTVD	396.2126	790.4185	f(134-140)	/	/
MES(phospho)TEVFTK	576.2371	1150.4617	f(141-149)	/	/
TKKTKLTE	474.7895	947.5651	f(148-155)	/	/
TKVIPYVRYL	417.9110	1250.7387	f(198-207)	Antimicrobial	Alvarez-Ordóñez et al., 2013

^aOne code letter was used for amino acid nomenclature.

^bThe observed mass is reported as $[M+nH]^{n+}$.

^cThe calculated mass is in Da.

^dPotential bioactivities were retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

Table S4. κ -casein-derived peptides identified in milk fermented with *Lactobacillus casei* PRA205 whole cells.

<i>Sequence^a</i>	<i>Observed mass (m/z)^b</i>	<i>Calculated mass^c</i>	<i>Fragment</i>	<i>Bioactivity^d</i>	<i>Reference</i>
FSDKIA	340.6720	679.3541	f(18-23)	/	/
KYIPIQY	462.7570	923.5116	f(24-30)	/	/
KYIPIQYVL	568.8331	1135.6641	f(24-32)	/	/
SRYPYGLN	528.7613	1055.5036	f(33-41)	/	/
YYQQKPV	463.2334	924.4705	f(42-48)	/	/
YYQQKPVAL	555.2851	1108.5917	f(42-50)	/	/
YYQQKPVALIN	668.8545	1335.7187	f(42-52)	/	/
YYQQKPVALINN	725.8879	1449.7616	f(42-53)	/	/
QKPVALINN	498.7884	995.5764	f(45-53)	/	/
NQFLPYPPYAKPA	786.4022	1570.7820	f(53-65)	/	/
QFLPYPPYAKPA	729.3616	1456.7391	f(54-65)	/	/
FLPYPPYAKPA	665.3372	1328.7805	f(55-65)	/	/
LPYPPYAKPA	591.8096	1181.6121	f(56-65)	/	/
YAKPA	275.1487	548.2958	f(61-65)	/	/
AVRSPA	300.6684	599.3391	f(66-71)	/	/
AVRSPAQIL	477.7794	953.5658	f(66-74)	/	/
AVRSPAQILQ	541.8009	1081.6244	f(66-75)	/	/
ARHPHPLS	351.1777	1050.5471	f(96-104)	/	/
ARHPHPLSF	400.2009	1197.6156	f(96-105)	/	/
ARHPHPLSFM	443.8951	1328.6560	f(96-106)	/	/
DKTEIPTIN	515.7586	1029.5342	f(116-123)	/	/
KTEIPTIN	458.2511	914.5073	f(117-123)	/	/
EIPTIN	686.3773	685.3646	f(118-123)	/	/
TIASGEPT	775.3848	774.3759	f(124-131)	/	/

VATLEDS(phospho)PE	520.7034	1039.4111	f(143-152)	/	/
VIESPPEIN	997.5065	996.5128	f(152-160)	/	/
SPPEIN	656.3215	655.3177	f(155-160)	/	/
SPPEINTVQ	984.5185	983.4924	f(155-163)	/	/
VTSTAV	577.3151	576.3119	f(164-169)	/	/

^aOne code letter was used for amino acid nomenclature.

^bThe observed mass is reported as $[M+nH]^{n+}$.

^cThe calculated mass is in Da.

^dPotential bioactivities were achieved retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

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Table S5. β -casein-derived peptides identified in milk fermented with *Lactobacillus rhamnosus* PRA331 whole cells^a.

<i>Sequence^b</i>	<i>Observed mass (m/z)^c</i>	<i>Calculated mass^d</i>	<i>Fragment</i>	<i>Bioactivity^e</i>	<i>Reference</i>
LNVPGEIVE	485.2516	968.5179	f(6-14)	ACEi	Gobbetti et al., 2000
NVPGEIVE	856.4256	855.4338	f(7-14)	/	/
SITRIN	352.1995	702.4024	f(22-27)	/	/
KKIEKF	396.7440	791.4905	f(28-33)	/	/
KKIEKFQS(phospho)E	608.8120	1215.5900	f(28-36)	/	/
DKIHPF	378.6905	755.3966	f(47-52)	ACEi	Gobbetti et al., 2000
DKIHPFA	414.2081	826.4337	f(47-53)	/	/
SLVYFPFGPIPN	1300.6991	1299.6863	f(57-68)	/	/
LVYFPFGPIPN	1213.6643	1212.6543	f(58-68)	/	/
VYFPFGPIPN	1100.5503	1099.5702	f(59-68)	ACEi Antioxidant, Opioid	Eisele et al., 2013
VYFPFGPIPNSLPQ	763.3809	1524.7977	f(59-72)	/	/
YFPFGPIPN	1001.5294	1000.5018	f(60-68)	ACEi DPPIV-inhibitor, Opioid	Saito et al., 2000
SLPQNIPPL	978.5704	977.5546	f(69-77)	/	/
SLPQNIPPLTQTPV	752.9436	1503.8297	f(69-82)	/	/
SLPQNIPPLTQTPVVVPPFLQPEVM	1371.2513	2740.4874	f(69-93)	/	/
QNIPPLTQTPV	604.3167	1206.6608	f(72-82)	/	/
QNIPPLTQTPVVVPPF	874.0031	1745.9716	f(72-87)	/	/
QNIPPLTQTPVVVPPFLQPE	738.7323	2213.2096	f(72-91)	/	/
QNIPPLTQTPVVVPPFLQPEVM	815.4550	2443.3185	f(72-93)	/	/
QNIPPLTQTPVVVPPFLQPEVMGVS	896.4847	2686.4404	f(69-96)	/	/
NIPPLTQTPV	540.3031	1078.6023	f(73-82)	ACEi	Gobbetti et al., 2000

NIPPLTQTPVVVPPF	809.9655	1617.9131	f(73-87)	/	/
NIPPLTQTPVVVPPFLQPEVM	1158.6488	2315.2599	f(73-93)	/	/
TQTPVVVPPF	542.7930	1083.5965	f(78-87)	/	/
TQTPVVVPPFL	599.3400	1196.6805	f(78-88)	/	/
TQTPVVVPPFLQPE	776.4225	1550.8345	f(78-91)	/	/
TQTPVVVPPFLQPEVM	891.4927	1780.9434	f(78-93)	/	/
TQTPVVVPPFLQPEVMGVS	1013.0422	2024.0653	f(78-96)	/	/
TQTPVVVPPFLQPEVMGVSKVKEAMAP	960.5032	2878.5337	f(78-104)	/	/
QTPVVVPPFLQPE	725.8957	1449.7868	f(79-91)	/	/
QTPVVVPPFLQPEVM	840.9478	1679.8957	f(79-93)	/	/
PVVVPPFLQPE	611.3391	1220.6805	f(81-91)	/	/
PVVVPPFLQPEVM	726.3960	1450.7894	f(81-93)	/	/
VVPPFLQPE	1025.5809	1024.5593	f(83-91)	/	/
VVPPFLQPEVM	1255.6756	1254.6682	f(83-93)	/	/
VPPFLQPEVM	578.7956	1155.5998	f(84-93)	/	/
PEVMGVSKVKEAMAPK	567.6384	1700.9074	f(90-105)	/	/
VMGSKVKEA	349.8603	1046.5794	f(92-101)	/	/
MAPKHKEMPFPKYPVEPF	725.0518	2172.0900	f(102-119)	/	/
APKHKEMPFPKYPVEPF	681.3265	2041.0495	f(103-119)	/	/
HKEMPFPKYPVEPF	582.6204	1744.8647	f(106-119)	/	/
EMPFPKYPVEPF	740.8436	1479.7108	f(108-119)	/	/
MPFPKYVVEP	602.8002	1203.5998	f(109-118)	/	/
MPFPKYVVEPF	676.3260	1350.6682	f(109-119)	/	/
MPFPKYVVEPFTE	791.3602	1580.7585	f(109-121)	/	/
NLHLPLP	402.2289	802.4701	f(132-138)	ACEi	Kohmura et al., 1989
NLHLPLPL	458.7739	915.5542	f(132-139)	/	/

NLHLPLPLL	515.3360	1028.6632	f(132-140)	ACEi	Robert et al., 2004
NLHLPLPLLQ	579.3461	1156.6968	f(132-141)	/	/
NLHLPLPLLQS	622.8557	1243.7288	f(132-142)	/	/
NLHLPLPLLQSW	715.8693	1429.8082	f(132-143)	/	/
LHLPLP	345.2061	688.4272	f(133-138)	ACEi	Kohmura et al., 1989
LHLPLPL	401.7568	801.5112	f(133-139)	ACEi	Quiros et al., 2007
LHLPLPLLQ	522.3205	1042.6539	f(133-141)	/	/
LHLPLPLLQS	565.8474	1129.6859	f(133-142)	/	/
LHLPLPLLQSW	658.8784	1315.7652	f(133-143)	/	/
HLPLPL	345.2061	688.4272	f(134-139)	/	/
HLPLPLLQSW	602.3478	1202.6812	f(134-143)	/	/
LPLPLLQ	793.5105	792.5109	f(135-141)	/	/
LPLPLLQSW	533.8107	1065.6223	f(135-143)	/	/
WMHQPHQPLPPTVMFPPQ	723.3596	2167.0496	f(143-160)	/	/
MHQPHQPLPPT	641.8081	1281.6288	f(144-154)	/	/
MHQPHQPLPPTVM	504.9035	1511.7377	f(144-156)	/	/
MHQPHQPLPPTVMFPPQ	661.3167	1980.9703	f(144-160)	/	/
HQPHQPLPPT	576.2926	1150.5883	f(145-154)	/	/
HQPHQPLPPTVM	461.2329	1380.6972	f(145-156)	/	/
HQPHQPLPPTVMFPPQ	617.6437	1849.9298	f(145-160)	/	/
FPPQSVL	787.4370	786.4272	f(157-163)	/	/
SQSKVLPVPQ	541.8019	1081.6132	f(166-175)	ACEi	Hayes et al., 2007
QSKVLPVPQ	498.2873	994.5811	f(167-175)	/	/
SKVLPVPQ	434.2558	866.5226	f(168-175)	ACEi	Yamamoto et al., 1994
KVLPVPQ	780.4975	779.4905	f(169-175)	ACEi	Maeno et al., 1996
VLPVPQ	652.3929	651.3956	f(170-175)	/	/

KAVPYPQ	401.7177	801.4385	f(176-182)	/	/
KAVPYPQRDMPI	707.8531	1413.7438	f(176-186)	/	/
RDMPIQAF	489.2344	976.4800	f(183-190)	ACEi	Yamamoto et al., 1994
RDMPIQAFLL	545.7702	1089.5641	f(183-191)	/	/
RDMPIQAFLL	602.3312	1202.6481	f(183-192)	/	/
LYQEPVL	861.4602	860.4644	f(192-198)	/	/
YQEPVL	748.3849	747.3803	f(193-198)	/	/
YQEPVLGPVRGPF	778.4100	1554.8195	f(193-206)	/	/
YQEPVLGPVRGPFPIIV	941.0424	1880.0560	f(193-209)	Immunomodulator	Boutrou et al., 2013
QEPVLGPVRGPF	686.8812	1391.7561	f(194-206)	/	/
QEPVLGPVRGPFPIIV	859.5135	1716.9927	f(194-209)	ACEi	Lu et al., 2016
EPVLGPVRGPF	632.8351	1263.6976	f(195-206)	ACEi	Hayes et al., 2007
EPVLGPVRGPFPIIV	795.4796	1588.9341	f(195-209)	/	/
VLGPVRGPFPIIV	682.4057	1362.8388	f(197-209)	/	/
LGPVRGPFPIIV	632.8861	1263.7703	f(198-209)	/	/
GPVRGPF	413.7160	825.4497	f(199-206)	/	/
GPVRGPFPII	526.7979	1051.6179	f(199-208)	/	/
GPVRGPFPIIV	576.3487	1150.6863	f(199-209)	/	/
RGPFPIIV	449.7698	897.5436	f(202-209)	/	/

^aAbbreviations are: ACEi, angiotensin converting enzyme-inhibitory; DPPIV, dipeptidyl peptidase IV.

^bOne code letter was used for amino acid nomenclature.

^cThe observed mass is reported as $[M+nH]^{n+}$.

^dThe calculated mass is in Da.

^ePotential bioactivities were achieved retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

Table S6. α S1-casein-derived peptides identified in milk fermented with *Lactobacillus rhamnosus* PRA331 whole cells^a.

<i>Sequence^b</i>	<i>Observed mass (m/z)^c</i>	<i>Calculated mass^d</i>	<i>Fragment</i>	<i>Bioactivity^e</i>	<i>Reference</i>
RPKHPIKH	338.1924	1011.6090	f(1-8)	/	/
RPKHPIKHQ	380.8905	1139.6676	f(1-9)	ACEi	Saito et al., 2000
RPKHPIKHQGLPQ	512.6139	1534.8844	f(1-13)	/	/
RPKHPIKHQGLPQEVLN	498.5303	1990.1224	f(1-17)	/	/
KHPIKHQ	444.2507	886.5137	f(3-9)	/	/
GLPQEVN	755.4085	754.4298	f(10-16)	/	/
GLPQEVNNE	499.7582	997.5080	f(10-18)	/	/
ENLLRF	396.2095	790.4337	f(18-24)	ACEi	Boutrou et al., 2013
FVAPFPE	806.4074	805.4010	f(24-30)	/	/
FVAPFPEVF	1052.5164	1051.5379	f(24-32)	ACEi	Boutrou et al., 2013
FVAPFPEVFGKE	683.8608	1365.6969	f(24-35)	/	/
VAPFPE	659.3349	658.3326	f(25-30)	/	/
VAPFPEVF	905.4909	904.4695	f(25-32)	ACEi	Boutrou et al., 2013
VAPFPEVFGK	545.8130	1089.5859	f(25-34)	/	/
VAPFPEVFGKE	610.3173	1218.6285	f(25-35)	/	/
APFPEVF	806.4074	805.4010	f(26-32)	/	/
APFPEVFGKE	560.7993	1119.5601	f(26-35)	/	/
VFGKEKVN	460.7541	919.5127	f(31-38)	/	/
KKYKVPQ	445.7685	889.5385	f(102-108)	/	/
KYKVPQ	381.7245	761.4436	f(103-108)	/	/
LEIVPN	684.3640	683.3854	f(109-114)	/	/
S(phospho)AEELRH	461.1787	920.3753	f(115-121)	/	/
S(phospho)AEELRHSM	570.2206	1138.4478	f(115-123)	/	/
KEGIHAQ	391.6973	781.4082	f(124-130)	/	/

APSFSDIPNPIGSENSE	880.9123	1759.7901	f(176-192)	/	/
FSDIPNPIGSE	588.2765	1174.5506	f(179-189)	/	/
FSDIPNPIGSEN	645.3093	1288.5935	f(179-190)	/	/
FSDIPNPIGSENSE	753.3208	1504.6682	f(179-192)	/	/
IPNPIGSENSE	578.7531	1155.5408	f(182-192)	/	/

^aAbbreviation is: ACEi, angiotensin converting enzyme-inhibitory.

^bOne code letter was used for amino acid nomenclature.

^cThe observed mass is reported as [M+nH]ⁿ⁺.

^dThe calculated mass is in Da.

^ePotential bioactivities were achieved retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

Table S7. α S2-casein-derived peptides identified in milk fermented with *Lactobacillus rhamnosus* PRA331.

<i>Sequence^a</i>	<i>Observed mass (m/z)^b</i>	<i>Calculated mass^c</i>	<i>Fragment</i>	<i>Bioactivity^d</i>	<i>Reference</i>
SIIS(phospho)QETYK	574.7617	1147.5162	f(13-21)	/	/
NAVPITPT	812.4463	811.4440	f(115-122)	/	/
NAVPITPTLN	520.2697	1038.5710	f(115-124)	/	/
NAVPITPTLNRE	662.8581	1323.7146	f(115-126)	/	/
AVPITPT	698.4069	697.4010	f(116-122)	/	/
AVPITPTLNRE	605.8419	1209.6717	f(116-126)	/	/
MES(phospho)TEVFTK	576.2277	1150.4617	f(141-149)	/	/
MES(phospho)TEVFTKK	640.2751	1278.5567	f(141-150)	/	/
TKVIPYVRYL	417.9150	1250.7387	f(198-207)	Antimicrobial	Alvarez-Ordóñez et al., 2013

^aOne code letter was used for amino acid nomenclature.

^bThe observed mass is reported as [M+nH]ⁿ⁺

^cThe calculated mass is in Da.

^dPotential bioactivities were achieved retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

Table S8. κ -casein-derived peptides identified in milk fermented with *Lactobacillus rhamnosus* PRA331 whole cells.

<i>Sequence^a</i>	<i>Observed mass (m/z)^b</i>	<i>Calculated mass^c</i>	<i>Fragment</i>	<i>Bioactivity^d</i>	<i>Reference</i>
FSDKIA	340.6721	679.3541	f(18-23)	/	/
KYIPIQY	462.7544	923.5116	f(24-30)	/	/
KYIPIQYVL	568.8299	1135.6641	f(24-32)	/	/
KYIPIQYVLS	612.3488	1222.6961	f(24-33)	/	/
SRYPYGLN	528.7591	1055.5036	f(33-41)	/	/
RYPYGLN	485.2353	968.4716	f(34-41)	/	/
YYQQKPVAL	555.2734	1108.5917	f(42-50)	/	/
YYQQKPVALIN	668.8605	1335.7187	f(42-52)	/	/
YYQQKPVALINN	725.8975	1449.7616	f(42-53)	/	/
QKPVALINN	562.8214	1123.6349	f(44-53)	/	/
QKPVALINN	498.7893	995.5764	f(45-53)	/	/
QFLPYPPYAKPA	729.3794	1456.7391	f(54-65)	/	/
FLPYPPYAKPA	665.3492	1328.6805	f(55-65)	/	/
LPYPPYAKPA	591.8004	1181.6121	f(56-65)	/	/
AVRSPA	300.6625	599.3391	f(66-71)	/	/
AVRSPAQIL	477.7849	953.5658	f(66-74)	/	/
AVRSPAQILQ	541.8019	1081.6244	f(66-75)	/	/
ARHPHPLS	351.1755	1050.5471	f(96-104)	/	/
ARHPHPLSFM	443.8816	1328.6560	f(96-106)	/	/
EIPTIN	686.3505	685.3646	f(118-123)	/	/
TIASGEPT	775.3862	774.3759	f(124-131)	/	/
VATLEDS(phospho)PE	520.7108	1039.4111	f(143-152)	/	/
VIESPPEIN	499.2569	996.5128	f(152-160)	/	/
SPPEIN	328.6530	655.3167	f(155-160)	/	/

SPPEINTVQ

492.7466

983.4924

f(155-163)

/

/

^aOne code letter was used for amino acid nomenclature.

^bThe observed mass is reported as $[M+nH]^{n+}$.

^cThe calculated mass is in Da.

^dPotential bioactivities were achieved retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

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